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PRINCIPAL INVESTIGATOR(S): Richard P. Junghans, Ph.D., M.D.

CONTRACTING ORGANIZATION: New England Deaconess Hospital  
Boston, Massachusetts 02215

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## INTRODUCTION.

Breast cancer presently kills more than 40,000 American women each year, second only to lung cancer as a cause of cancer death in women [1]. There is evidence for a small advantage in long term survival with chemotherapies in adjuvant settings [2], but there is little convincing evidence for systemic cures with any therapy where known residual metastatic disease exists. Thus, there is an enduring need for new approaches to treatment in breast cancer. In the search for such new approaches, it has been proposed by Lippman that "the most appropriate protein targets may emerge from a consideration of prognostic variables ... shown to be of value in clinical practice" [3]. The present work builds on this premise, to explore the molecular basis for the apparently improved survival in plasma cell-infiltrated medullary and other breast carcinomas, and specifically to describe the new breast carcinoma-associated proteins that elicit this response.

Medullary carcinomas (MC) are diagnosed in up to 5-7% of breast cancers [4]. MC is circumscribed grossly and microscopically without encapsulation, but its appearance is otherwise highly ominous, with large cells, abundant cytoplasm, large bizarre nuclei and frequent mitoses. Virtually all are histologic grade III, usually the worst prognostically, and they display a high degree of aneuploidy and typically lack hormone receptors. Yet patients with MC often do better than predicted for size and grade. Tumor is infiltrated and surrounded with lymphocytes and plasma cells; in its most exuberant expression, it was classically designated "medullary carcinoma with lymphoid stroma", prompting the off-stated impression that this tumor may be regulated by a host immune response.

It is our aim to derive antibodies from these plasma cells with specificity for malignant breast tissues using molecular techniques based on phage display libraries [5]. These powerful procedures can reconstruct immunoglobulin reactivities, even when sparsely expressed. Moreover, this approach maximally exploits the enrichment for reactivity and affinity for the tumor tissue which is inherent to the mature plasma cells, whose abundant Ig mRNA dominates over less mature and less affinity-selected B cells during the molecular cloning. The procedures are rapid and their products are truly human antibodies — without the attendant problems and limitations of human antibody technology (which will not immortalize plasma cells) [6].

## STUDY PROGRESS

In the original submission, we confirmed the IgG predominance of plasma cell infiltrations in one MCB specimen, demonstrated procedures for tumor disaggregation and cell separation, described a breast cell culture strategy we used, and obtained the HTB24 (MB157) medullary carcinoma cell line. We showed that we could successfully amplify the genes from breast tumor plasma cells by PCR and that the pattern of  $\lambda$  amplifications suggested an oligoclonality to the Ig genes in the tumor, suggesting in turn a restricted immune response representation. In the period since the original proposal, we have obtained two additional medullary carcinoma samples for a total of three; we have prepared  $\gamma 1, \kappa$  and  $\gamma 1, \lambda$  libraries into M13 phage for two of these, with  $> 10^6$  members, well in excess of what we will need for effective representation in a restricted antibody library. We have devised procedure improvements to assess library quality and to streamline screening that will increase the power of the phage display method [7].

In a library of high diversity, there is no repetition of clones in any practical sized sample. In hyperimmunized individuals, antitetanus antibodies were present in a total B cell library in only only 1:1000 to 1:5000 clones, and only 2/8 selected *positive* clones that were sequenced showed the same V gene usage [8,9]. In a further study with influenza-immunized mice, a dominant H chain and a dominant L chain were present in maximum proportions of 1:200 and 1:1000 in a total library, respectively [10]. Hence, any recurrence of genes in a limited random sample will signal that the library is highly focussed and restricted.

In Ig sequencing of two patient MC samples, we were surprised by the already dramatic focus in the libraries, in the absence of *in vitro* panning. There was an apparent reiteration of sequences in these derived and diversified from single B cell clones. The possibility of lab *PCR* or *Fab-phage contaminants* was ruled out by comparing against all other cloned Ig genes and phage-Fab in our laboratory. The possibility of a repeated *plasmid clone* was ruled out by the presence of different partners with each of the repeated chains, and by the different mutations present in the repeated clones in the different isolates. That the repeated clones of a given group are from a single original B cell is indicated by the minigene (CDR3) patterns in each group. We interpret the presence of one repeated clone among the  $V\kappa$  sequences and two repeated clones among the  $VH$  as indicating that one of the original  $VL$  chains is missing; we expect that the other will be among the  $V\lambda$ , to be examined shortly. Until single cell PCR studies are performed, we will not know the original  $VHVL$  pairings, but we may be able to surmise the general pairs by those showing best antigen reactivity, which should be apparent after sequencing the clones enriched on cell panning. These several clones that appear to represent the repertoire focus, as well as the whole  $IgG, \kappa$  and  $IgG, \lambda$  libraries were recently tested in a Her2/neu ELISA,

and none was reactive, therefore establishing that the focussed part of the repertoire is not in response to Her2/neu. Early data including 5 VH and 5 V $\kappa$  from a second patient's tumor clearly show reiteration as well (2/5 VH; 3/5 V $\kappa$ ), but more sequences are needed to comment on commonality of gene usage between donors. Repetition of V genes between patients will be strong evidence for a common antigen, but divergent gene usage will not rule it out.

These results support one of our premises that the tissue will present a highly restricted antibody diversity as expected from a limited, specific response within the tissue. Because our antigen is unknown, it is essential that our panning methods are optimized so that negative results are meaningful and so that enrichments of positive clones are efficient, even with low antigen expression. Particle [10] and cell-based [11; A Griffiths, G Winter, unpubl.res.] selections have been applied in other systems, but these were with known antigens present in high concentrations. Furthermore, to our knowledge, prior cellular panning methods have used only erythrocytes, which are physically very different from plasma membrane cells of carcinomas which we wish to study.

We accordingly investigated optimal conditions for these pannings [J Watters, P Telleman & R Junghans, in prepn.], which required a model system with a cloned Fab-phage reactive with a cell surface molecule. We previously cloned an anti-idiotypic Fab-phage from a combinatorial phage library derived from a patient treated with murine anti-Tac antibody [7]. This was tested against hybridoma cells expressing the anti-Tac antibody (HD245, gift of T Waldmann). This showed reactivity of Fab-phage by flow cytometry that was specific because excess unlabeled anti-Tac antibody suppressed phage binding to cells whereas a non-specific phage against tetanus toxoid (TT) did not react with HD245.

With confirmation of surface expression of antibody on the hybridoma cell line, we proceeded to tests with panning and phage titer assessment. A ten-fold enrichment of anti-id phage titer was observed relative to non-specific phage (TT) in one cycle of panning that was suppressed to non-specific levels in the presence of competing "antigen" (anti-Tac antibody) [J Watters, P Telleman & R Junghans, in prepn.]. Several features of the assay were investigated. Under optimal conditions, panning in this system led to 20- to 40-fold enrichments of specific over non-specific phage with each cycle. This is less than in pannings against purified proteins with standard methods [5], but sufficient for focussed, reduced-diversity libraries. These optimizations were recently completed.

In the few days prior to this submission, we applied these techniques to perform panning of our two MC libraries (pooling IgG  $\kappa$  and  $\lambda$  libraries) against HTB24 cells, the only available MC cell line. As a negative control, we used the tetanus toxoid (TT) Fab-phage clone from an unrelated library. This showed 10- to 20-fold enrichments after a single cycle of binding to HTB24 relative to the TT Fab-phage in two separate experiments on different days. This observation is consistent with a library that is already highly enriched for antigen reactivity,

which we estimate as 60% relative to the pooled VHVL libraries of one patient for whom extensive sequence data is available. This observation is an extremely important result. It confirms surface expression of the putative neo-antigen, and enables all of the immunoprecipitation and gene cloning approaches in Methods. By sequencing the clones out of these pannings, it should be apparent which VHVL pairs are reactive and presumably derived from canonical initial B cell clones.

In a parallel effort to develop MCB cell lines, we tested two tumors for growth in scid mice, including one test with irradiated, estrogen-supplemented animals, but only one tumor showed initial growth that then regressed. It is known that malignant effusions are the best source of cells for generating tumor cell lines, and this was the source for the world's only MCB cell line. We have accordingly initiated a national search for pleural effusions from patients with MCB diagnosis with announcements in the *Journal of Clinical Oncology*, *American Journal of Surgical Pathology*, *Oncology News International*, and other sources. We have also made contingency plans that would permit us to retrieve retrospectively the original antibody reactivities of those patients using old paraffin cell blocks and glass slides of such patients presenting with effusions long after their original presentation. As RNA will likely be too degraded in old samples from the plasma-cell infiltrated primary tumor tissue, we have designed a new strategy and new primers for PCR from the DNA of these tissues that will reconstruct the library based on the V(D)J recombinations at the immunoglobulin locus in the cells.

## CONCLUSIONS.

Our sequence data support the premises that the MCB tissue will present a highly restricted antibody diversity as expected from a limited, specific response within the tissue. Furthermore, preliminary data indicated binding of unselected phage-Fab clones to HTB24 cells, confirming cell surface expression of the putative neo-antigens on these cells.

In the future these studies will enable (1) isolation of breast carcinoma-reactive Fab from plasma cell-infiltrated breast tissues, and (2) identification of breast carcinoma neo-antigens eliciting the plasma cell responses. Followup studies will build on these results to provide a detailed biochemical and molecular characterization of these neo-proteins, with further efforts to elucidate their role in tumorigenesis and potential for targeting through rationally designed strategies based on features of their actions.

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